BIOLOGICALLY ACTIVE PEPTIDES FROM THE PERICARDIAL ORGANS OF THE CRAB CANCER BOREALIS [1]

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Pericardial organs are neurosecretory structures located in the pericardial cavity of decapod and stomatopod crustaceans. Alexandrowicz (1952, 1953) was the first to figure these structures and, with Carlisle, to show that low concentrations of aqueous extracts acted as a potent cardio-excitor (Alexandrowicz and Carlisle, 1953). Extracts produced a marked increase in amplitude and frequency in all the crustacean species studied except *Maia squinado,* which exhibited an increase in amplitude, but a decrease in frequency. These investigators also found that blood collected from the pericardial cavity produced excitation similar to that of pericardial organ extracts. Cooke (unpublished data) has demonstrated that cardio-excitor hormone is released by electrical stimulation of the pericardial organs.

Carlisle (1956) reported finding two active areas when pericardial organ extracts were subjected to paper chromatography, both of which gave color reactions indicative of indole alkylamines. One of the active areas was destroyed by ortho-diphenol oxidase more rapidly than the other, an action that led Carlisle to believe the latter to be a precursor. Carlisle speculated that the active substance might be an ortho-dihydroxytryptamine, and later considered 5,6-dihydroxytryptamine the most likely possibility (Carlisle and Knowles, 1959).

Maynard and Welsh (1959) found 5-hydroxytryptamine to be present in pericardial organ extracts, but at concentrations too low to account for the marked activity of the extract. These authors demonstrated that pericardial organ extracts could be inactivated by trypsin and chymotrypsin, and that the active principle was (1) dialyzable, (2) soluble in aqueous and alcoholic solvents, but only sparingly soluble in acetone, and (3) heat-stable at neutral and acid pH. They tentatively identified the neurosecretory material as a peptide.

The purpose of this paper is to present further data from studies on the nature of the pericardial organ neurohormone.

Materials and Methods

Cancer borealis and *Cancer irroratus* were used as a source of pericardial organs; lobster hearts were used for assay. Animals were obtained from the Crab and Lobster Company, Boothbay Harbor, Me., or from local sources. Pericardial organs were removed by the method of Maynard and Welsh (1959) and either used

[1] This research was supported in part by a predoctoral fellowship from the National Institutes of Health.

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immediately or freeze-dried and stored in a desiccator at 10° C. Pericardial organs stored by this method for more than one year were still active.

Extracts of pericardial organs were made by grinding either the fresh or freeze-dried tissue in a glass homogenizer with the appropriate solvent. Aqueous extracts were heated at 100° C. for a few minutes and centrifuged, the sediment being washed several times and the washes pooled with the supernatant. For paper chromatography or paper electrophoresis, extracts were generally made in 95% ethanol and washed with petroleum ether.

All extracts not made in *Homarus* perfusion fluid (Welsh and Smith, 1960, after Cole) were lyophilized or taken to dryness under reduced pressure. The residue was redissolved in perfusion fluid before assaying. Extracts were tested on a perfused isolated lobster heart which was kept at a constant temperature. Although individual experiments were done at constant temperatures, the range of temperatures during the course of these experiments was 15° to 20° C. A small volume (0.5 ml.) of the extract was introduced into the perfusion medium by syringe without disturbing the rate of flow of the fluid, and the action of the heart was recorded on a smoked drum.

Paper electrophoresis of pericardial organ extracts was carried out utilizing Whatman 3 MM paper and buffers (acetate, phosphate, Tris, borate) with a pH range of 2.6 to 10.1 and ionic strength of 0.05. Voltage was usually 11.4 V./cm., although runs were also made at 5 V./cm. The extract, from 1–10 pairs of pericardial organs, was taken down to dryness under reduced pressure. It was then redissolved in a small amount (50–100 μl.) of 95% ethanol and streaked at the center of a prewashed paper. Lengths of run were 1 to 24 hours, after which papers were either air-dried, oven-dried at 60° C. and cut into sections, or cut while still wet. The latter method was the method of choice. The section which included the origin and one section to either side of the origin was of 1 cm. width, while all other sections were 2 cm. The sections were eluted into 3 ml. of solvent, freeze-dried if run in volatile buffers, and assayed. For staining, strips were cut from the long axis of the paper before sections were made.

Chromatography of pericardial organ extracts was performed by the ascending one-dimensional method, utilizing n-butanol/acetic/water (60:15:25) and Whatman 3 MM paper. Papers were pre-washed with distilled water and ethanol. Development was usually 12–17 hours at room temperature. An extract from 4 to 20 pairs of pericardial organs was applied as a streak and developed, after which a longitudinal strip was cut from the paper and sprayed with various staining reagents. In this way the area of biological activity was correlated with staining reaction.

Eluates of the areas which produced activity and which were ninhydrin-positive were hydrolyzed by the method of Consden, Gordon and Martin (1947). The hydrolysates were spotted on Whatman No. 1 paper, along with amino acid standards, developed in n-butanol/acetic/water (60:15:25) or n-butanol/pyridine/water (1:1:1). All hydrolysis experiments were done with pericardial organs from *Cancer borealis*.

The confirmation of acidic and basic amino acid residues was accomplished with paper electrophoresis employing Schleicher and Schuell number 589 paper and 0.05 *M* acetate buffer at pH 4.7. The hydrolysate, after being dried over NaOH,

was taken up in dilute NH_4OH and applied to the center of the paper. A paper with lysine, glycine and glutamic acid applied at the origin was run simultaneously with the hydrolysate as a standard.

Results

Inactivation of pericardial organ active substance was accomplished by incubating extracts made in *Homarus* perfusion fluid (pH 7.6) with crystalline trypsin or chymotrypsin (approximately 10 μg./ml. extract) for one hour at 37° C. The enzymes alone had little, if any, effect on the heart. Heated enzyme incubated with pericardial organ extract did not affect the activity. These results confirm those obtained by Maynard and Welsh (1959). Trypsin was used periodically to inactivate extracts and eluates from paper electrophoresis and chromatography. This gave assurance that the activity was due to pericardial organ cardio-excitor substance.

A typical assay of a pericardial organ extract after electrophoresis is shown in Figure 1. As was true in most assays, the activity was spread over several centimeters with a major peak at one fraction. Where activity was found to be equally distributed between fractions, it is assumed that the fraction boundary line cut the activity peak in two.

The data in Figure 2 demonstrate that the isoelectric point of the active material is near pH 3, and an increase in pH above this point causes a corresponding increase

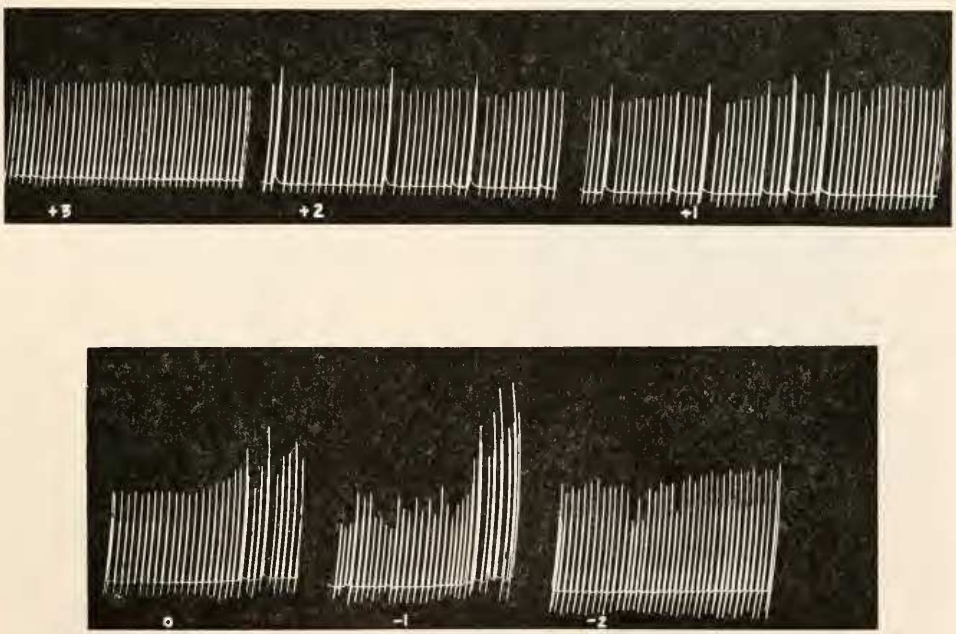

Figure 1. A typical assay of pericardial organ extract after paper electrophoresis. Extract of four pairs of *Cancer borealis* pericardial organs. Each fraction eluted into 3.0 ml. perfusion fluid and assayed on a perfused isolated lobster heart. Tris buffer, pH 7.7, 14 hours, 150 V.

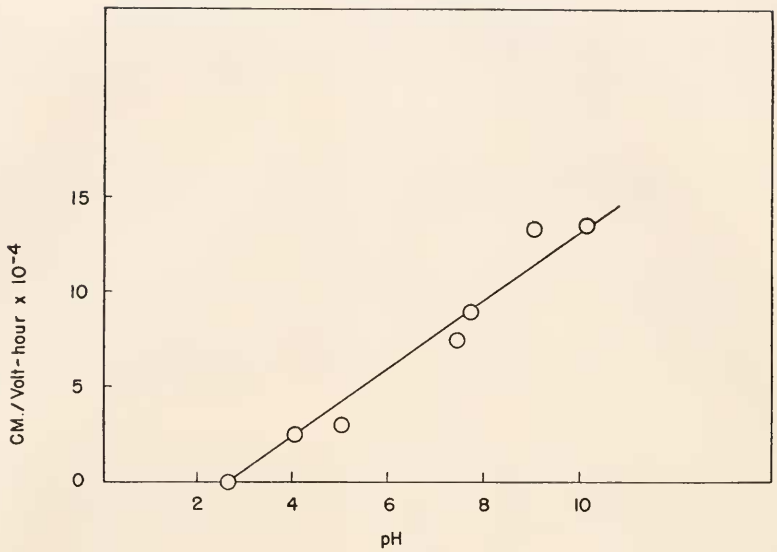

FIGURE 2. Isoelectric point of pericardial organ cardio-excitor material. Buffer ionic strength 0.05.

in migration toward the anode. A series of electrophoresis runs at pH 9.0, of increasing lengths of time, was made in an attempt to correlate staining reaction with migration of activity. Of the numerous reagents applied, only ninhydrin consistently stained the active area.

Tests of eluates of chromatogrammed pericardial organ extracts showed activity from two areas. This activity resembled but did not equal that produced by crude extracts. Such activity could be inactivated by trypsin. The activity was localized to two ninhydrin-positive areas (Fig. 3).

Chromatography of acid hydrolysates of the two active areas showed that these were two peptides of similar nature. These peptides have been given the designation peptides A and B. Hydrolysates of these peptides were subjected to paper chromatography and paper electrophoresis under similar conditions. These revealed the presence of glutamic acid and a positively charged amino acid (lysine or arginine), as well as the presence of neutral amino acids. Based on staining intensity, the glutamic acid was present in greater concentration than the positively charged amino acid. One other acidic residue, which had a migration rate slightly higher than that of glutamic acid, was also present in low concentration (Fig. 4). The neutral amino acids were categorized by their R_f values and comparison with standards in two chromatographic solvents, and were tentatively identified as serine in both peptides, tyrosine in peptide A, and methionine in peptide B. Each peptide contained one other residue which appeared in the region of valine or phenylalanine.

In order to demonstrate that one was dealing with the same material in electrophoresis and chromatography, a pericardial organ extract was subjected to electrophoresis and the area of activity determined by assay. A matching area from a simultaneously run paper was then eluted and subjected to paper chromatography. Examination showed that the two active, ninhydrin-positive areas present in the

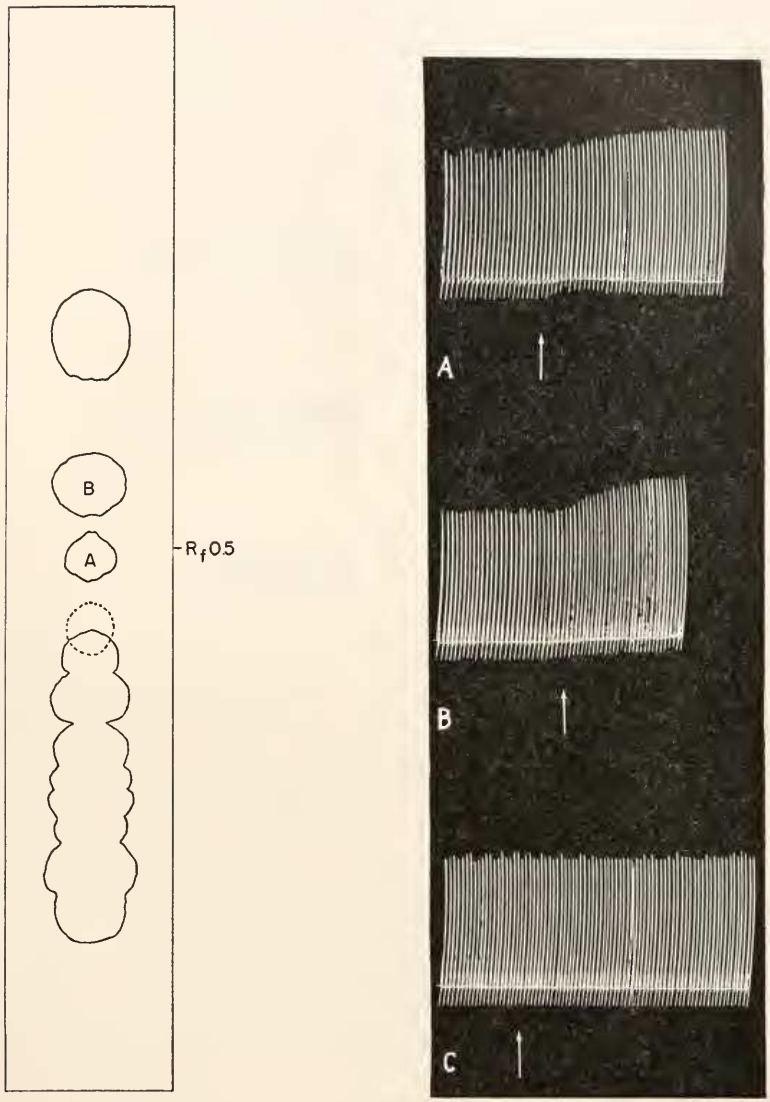

FIGURE 3. Chromatography and assay of an extract of 20 pairs of pericardial organs; n-butanol/acetic/water; 14 hours. Ninhydrin stain. A and B are active areas, C is control. Arrow marks point at which eluate reaches heart.

chromatogrammed pericardial organ extract were also present in eluates of active areas following electrophoresis.

DISCUSSION

Maynard and Maynard (1962) estimate that the neurosecretory granules may form 2% or less of the volume of the trunks of brachyuran pericardial organs. Since the average dry weight of freeze-dried pericardial organs from *Cancer borealis* was approximately 0.6 mg./animal, the amount of material in the neurosecretory

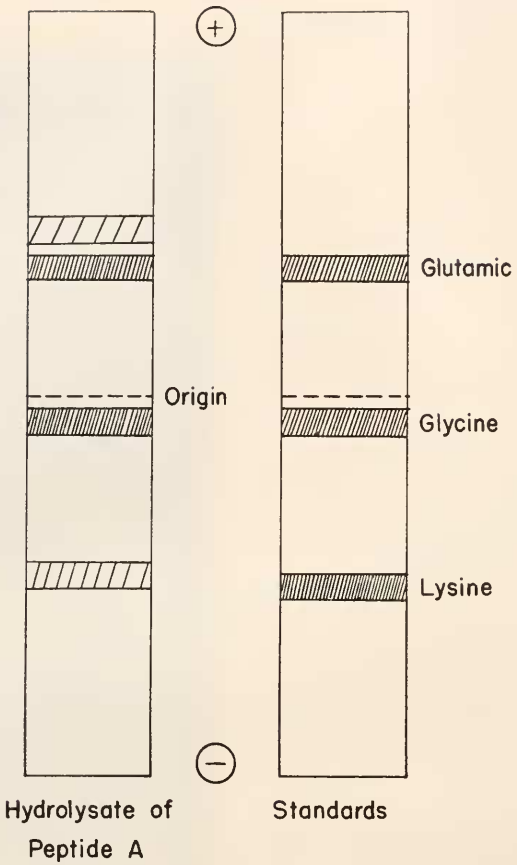

FIGURE 4. Electrophoresis of hydrolysate of peptide A. Acetate buffer, pH 4.7, ionic strength 0.05, 200 V., 4 hours.

granules can be calculated to be approximately 12 μg./animal. Although there is disagreement as to whether neurohormones are attached to a carrier protein or whether they are split off from a parent molecule (Bern, 1962), large protein molecules appear to be present in most, if not all, neurosecretory granules. The presence of such a protein, along with other components, would make the figure for actual neurohormone material present in the pericardial organ somewhat less than the calculated 12 μg./animal.

Pericardial organ extracts subjected to paper electrophoresis and paper chromatography apparently lose some activity. Smyth (1959) also noted this with crayfish pericardial organs, and found that developing chromatograms in an atmosphere of nitrogen or in the presence of ascorbic acid did not prevent loss of activity. With paper electrophoresis there was a correlation between the length of run and activity recovered, *i.e.*, short runs retained proportionally more activity.

The fact that there are two active components is not surprising. Carlisle (1956) reported finding two areas of activity after chromatography of pericardial organ extract. Of interest in this respect is the report by Alexandrowicz (1953) in which

he distinguishes two types of neurosecretory fibers contributing to the pericardial organ system of *Squilla*. Electron microscopy (Knowles, 1960) shows two types of neurosecretory granules in pericardial organs of *Squilla*, differing in size and internal structure. More recently, Maynard (1961) demonstrated the presence of three types of secretory cells contributing fibers to the pericardial organ-anterior ramification complex in several species of decapod crustaceans. When examined by electron microscopy, *Cancer* pericardial organs showed neurosecretory granules of only one size, but the other brachyuran genera examined contained granules of two or three size groups (Maynard and Maynard, 1962).

The data indicate that the active compounds extracted from pericardial organs are acidic peptides with an isoelectric point near pH 3. The presence of proportionally more glutaminyl residues than positively charged residue (lysine or arginine) fits well with this conclusion. The proposed amino acid composition of the peptides fits the requirements for enzymatic degradation by trypsin and chymotrypsin, since trypsin is specific for lysine or arginine linkages, while chymotrypsin is specific for aromatic, as well as leucyl, methionyl, arginyl, and glutaminyl bonds.

The identification of the neutral and basic amino acid residues has been made on a tentative basis. An examination of peptides A and B at this time indicates the possibility of a single amino acid difference. A small difference, especially in the neutral amino acids, would account for the inability to separate the peptides by means of paper electrophoresis. The residue exhibiting slightly higher negative charge than glutamic acid has not been identified. It is possible that a non-amino acid residue is present with the peptide. Smyth (1959) noted that after chromatography of crayfish pericardial organs in methanol/water/pyridine the active area stained lightly with ammoniacal silver nitrate as well as with ninhydrin.

Tryptophan analysis was not feasible because of the small amount of active material present in pericardial organs. However, it should be pointed out that pericardial organ extracts and 5-hydroxytryptamine have a qualitatively similar action on the decapod heart (Maynard and Welsh, 1959; Cooke, 1962), and this similarity may be due to the presence of tryptophan in the peptide.

Carlisle has considered 5,6-dihydroxytryptamine as the most likely possibility for the pericardial organ neurohormone. Such a compound has been shown to be extremely labile, exhibiting spectral shifts after 30 minutes in aqueous solution at pH 7, and after 5 minutes at pH 8 (Schlossberger and Kuch, 1960). There have been no published data to show that such a compound, in fact, exists in pericardial organs of decapod crustaceans, although the possibility is not discounted. If present, it may be acting at a different site on the cardiac ganglion, as has been demonstrated for 5-hydroxytryptamine (Cooke, 1962). The rate at which pericardial organ extracts used in this study lose activity, as well as other criteria, indicates that the activity of these extracts is not due entirely to 5,6-dihydroxytryptamine.

It is interesting to note that enzyme inactivation studies of crustacean chromatophorotropins and retinal pigment hormones show that all these neurosecretory products are inactivated by either trypsin or chymotrypsin or both of these enzymes (Knowles, Carlisle and Dupont-Raabe, 1956; Pérez-González, 1957; Kleinholz, Esper, Johnson and Kimball, 1961). It is well known that the neurosecretory hormones of the vertebrate hypothalamic-neurohypophyseal system are peptides. Future research may show that one of the main functions of neurosecretory systems is the production of biologically active peptides.

I wish to express my appreciation to Professor John H. Welsh for his continued guidance and interest in this problem, to Dr. Russell F. Doolittle for his invaluable advice, and to Misses Carolyn Sharpe and Joyce Zipf for help in the dissections.

Summary

1. The isoelectric point of pericardial organ cardio-excitor material, determined by paper electrophoresis, is near pH 3.

2. Paper chromatography demonstrates two active compounds in pericardial organ extracts which stain with ninhydrin and upon acid hydrolysis are identified as peptides. These peptides differ in composition by only a small number of residues.

3. The peptides contain proportionally more glutaminyl residues than positively charged residue (lysine or arginine), one other negatively charged residue, and a small number of neutral amino acids.

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